METHOD OF DETECTING β3 ADRENALINE RECEPTOR MUTANT GENE AND NUCLEIC ACID PROBE AND KIT THEREFOR

Technical Field

The present invention relates to method for detecting a β3-adrenergic receptor mutant gene, and a nucleic acid probe and a kit therefor.

Background Art

The β3-adrenergic receptor (B3AR) plays a major part in lypolysis in white adipose cells and heat generation in brown adipose cells. The existence of the mutation replacing tryptophan at position 64 in the amino acid sequence of B3AR with arginine (Trp64Arg) is said to reduce the resting metabolic rate by 200 kcal, and to be involved in obesity with abdominal fat and in insulin resistance.

If the mutation resulting in the Trp64Arg mutation in B3AR (also referred to as "B3AR Trp64Arg mutation") exists, a recognition site of a restriction enzyme emerges at the position of the mutation. Therefore, the mutation can be detected by a method of amplifying DNA by PCR so that a portion including the mutation position should be amplified, digesting the amplification product with a restriction enzyme and determining whether the DNA has been digested or not by electrophoresis (PCR-RFLP) (for example, refer to The Japanese Journal of Clinical Pathology, vol. 44, 8, pp.778-782, 1996).

Because PCR amplifies templates of several molecules several billion times, even a trace amount of contaminant may cause a false positive or false negative result. In PCR-RFLP, the amplification product needs to be collected and subjected to a treatment with a restriction enzyme

after PCR, and therefore the amplification product may contaminate the subsequent reaction system. Accordingly, a false positive or false negative result may be obtained.

Further, DNA is treated with a restriction enzyme and then subjected to electrophoresis after completion of PCR. Therefore, time required for the detection becomes extremely long. In addition, because the procedure is complicated, automatization is difficult.

Furthermore, a method is generally known in which a region containing a mutation is amplified by PCR, then a melting curve analysis is performed by using a nucleic acid probe labeled with a fluorescent dye, and the mutation is analyzed on the basis of the result of the melting curve analysis (Clinical Chemistry, vol. 46, 5, pp.631-635, 2000; Japanese Patent Application Laid-open (Kokai) No. 2002-119291).

Disclosure of the Invention

An object of the present invention is to identify a quenching probe effective for the detection of the B3AR Trp64Arg mutation and thereby provide a method for detecting the B3AR Trp64Arg mutation and a kit therefor.

The literature concerning the aforementioned method of using a probe only teaches that, concerning the design of the probe, the probe should be designed so that, when a quenching probe having an end labeled with a fluorescent dye hybridizes with a target nucleic acid, two or more nucleotide pairs of the probe-nucleic acid hybrid should form at least one pair of G and C in the end portion. With regard to the B3AR Trp64Arg mutation, the inventors of the present invention designed a quenching probe satisfying the aforementioned condition and attempted the detection. However, no quenching probe that enabled detection was easily obtained.

The inventors of the present invention found that by

designing a quenching probe based on a specific region containing the B3AR Trp64Arg mutation, the B3AR Trp64Arg mutation could be detected by a melting curve analysis using the quenching probe.

The present invention provides the followings.

(1) A nucleic acid probe of which end is labeled with a fluorescent dye, and in which fluorescence of the fluorescent dye decreases upon hybridization, wherein the nucleic acid probe has a nucleotide sequence starting from the nucleotide number 183 in the nucleotide sequence of SEQ ID NO: 1 and having a length of 8 to 30 nucleotides, and the 5′ end of the probe is labeled with the fluorescent dye, or the nucleic acid probe has a nucleotide sequence ending at the nucleotide number 196 in the nucleotide sequence of SEQ ID NO: 2 and having a length of 7 to 30 nucleotides, and the 3′ end of the probe is labeled with the fluorescent dye.

(2) The nucleic acid probe according to (1), wherein the nucleic acid probe has any one of the nucleotide sequences of SEQ ID NOS: 8 to 12.

(3) A method for detecting a mutation comprising performing a melting curve analysis for a nucleic acid having a single nucleotide polymorphism site by using a nucleic acid probe labeled with a fluorescent dye and measuring fluorescence of the fluorescent dye, and detecting the mutation on the basis of the result of the melting curve analysis, wherein the single nucleotide polymorphism is a mutation in a nucleotide sequence in a nucleic acid encoding a β3-adrenergic receptor, resulting in a mutation replacing tryptophan at position 64 in an amino acid sequence of the β3-adrenergic receptor with arginine, and the nucleic acid probe is the nucleic acid probe as defined in (1) or (2).

(4) The method according to (3), wherein a region containing a single nucleotide polymorphism site in a

nucleic acid contained in a sample is amplified to obtain the nucleic acid showing the single nucleotide polymorphism.

(5) The method according to (4), wherein the amplification is performed by a method of using a DNA polymerase.

(6) The method according to (5), wherein the amplification is performed in the presence of a nucleic acid probe.

(7) A kit for the method as defined in (3), which comprises a nucleic acid probe of which end is labeled with a fluorescent dye, and in which fluorescence of the fluorescent dye decreases upon hybridization, wherein the nucleic acid probe has a nucleotide sequence starting from the nucleotide number 183 in the nucleotide sequence of SEQ ID NO: 1 and having a length of 8 to 30 nucleotides, and the 5′ end of the probe is labeled with the fluorescent dye, or the nucleic acid probe has a nucleotide sequence ending at the nucleotide number 196 in the nucleotide sequence of SEQ ID NO: 2 and having a length of 7 to 30 nucleotides, and the 3′ end of the probe is labeled with the fluorescent dye.

(8) The kit according to (7), wherein the nucleic acid probe has any one of the nucleotide sequences of SEQ ID NOS: 8 to 12.

(9) The kit according to (7) or (8), which further comprises a primer for amplifying a region containing a mutation in a nucleotide sequence in a nucleic acid encoding a β3-adrenergic receptor, resulting in a mutation replacing tryptophan at position 64 in an amino acid sequence of the β3-adrenergic receptor with arginine, by a method of using a DNA polymerase.

Brief Description of the Drawings

Fig. 1 shows positions of quenching probes that cannot identify a mutation.

Fig. 2 shows positions of quenching probes that can

identify a mutation.

Fig. 3 shows sensitivity of the method of Example 1 (using probe 5FL-wt-1-16) with respect to the absolute amount of genomic DNA.

Fig. 4 shows reproducibility of the method of Example 1 (using probe 5FL-wt-1-16).

Fig. 5 shows sensitivity of the method of Example 1 (using probe 3T-mt-2-20) with respect to the absolute amount of genomic DNA.

Fig. 6 shows reproducibility of the method of Example 1 (using 3T-mt-2-20).

Best Mode for Carrying out the Invention

<1> Probe of the present invention and detection method of the present invention

The probe of the present invention is a nucleic acid probe of which end is labeled with a fluorescent dye, and in which fluorescence of the fluorescent dye decreases upon hybridization, wherein the probe has a nucleotide sequence starting from the nucleotide number 183 in the nucleotide sequence of SEQ ID NO: 1 and having a length of 8 to 30 nucleotides, and the 5′ end of the probe is labeled with the fluorescent dye, or the probe has a nucleotide sequence ending at the nucleotide number 196 in the nucleotide sequence of SEQ ID NO: 2 and having a length of 7 to 30 nucleotides, and the 3′ end of the probe is labeled with the fluorescent dye.

The probe of the present invention may be similar to the quenching probe described in Patent Document 1 except that it has a nucleotide sequence starting from the nucleotide number 183 in the nucleotide sequence of SEQ ID NO: 1 (sequence having the wild type nucleotide in the B3AR Trp64Arg mutation) and having a length of 8 to 30 nucleotides, or a nucleotide sequence ending at the nucleotide number 196 in the nucleotide sequence of SEQ ID

NO: 2 (sequence having the mutant type nucleotide in the B3AR Trp64Arg mutation) and having a length of 7 to 30 nucleotides. Examples of the nucleotide sequence of the quenching probe used in the present invention include the nucleotide sequences of SEQ ID NOS: 8 to 12. As the fluorescent dye, those described in Patent Document 1 can be used, and specific examples thereof include FAM (trademark), TAMRA (trademark), BODIPY (trademark) FL and so forth. The fluorescent dye can be bound to an oligonucleotide in an ordinary manner, for example, by the method described in Patent Document 1.

The detection method of the present invention is a method for detecting a mutation by performing a melting curve analysis for a nucleic acid having a single nucleotide polymorphism site by using a nucleic acid probe labeled with a fluorescent dye and measuring fluorescence of the fluorescent dye, and detecting the mutation on the basis of the result of the melting curve analysis, and characterized in that the single nucleotide polymorphism is the B3AR Trp64Arg mutation, and the nucleic acid probe is the probe of the present invention.

The detection method of the present invention can be performed according to usual methods for nucleic acid amplification and melting curve analysis (Tm analysis) except that a region containing the B3AR Trp64Arg mutation in a DNA encoding B3AR is amplified, and the probe of the present invention is used.

As the method for nucleic acid amplification, a method of using a polymerase is preferred, and examples thereof include PCR, ICAN, LAMP and so forth. When amplification is performed by a method using a polymerase, amplification is preferably performed in the presence of the probe of the present invention. The reaction conditions of the amplification and others can be easily adjusted depending on the used probe by those skilled in

the art. In this method, only Tm of the probe is analyzed after amplification of a nucleic acid, and therefore it is not necessary to handle the amplification product after completion of the reaction. Thus, there is no risk of contamination with the amplification product. Further, because the detection is performed with the same equipment as required for the amplification, it is not even necessary to move a vessel. Therefore, automatization of the method is also easy.

The method will be further explained below by referring, as an example, to a case of using PCR. The primer pair used for PCR can be designed in the same manner as in a method for designing a primer pair in usual PCR except that it is designed so that a region to which the probe of the present invention is hybridizable should be amplified. The length and Tm of the primers are usually 10- to 40-mer and 40 to 70°C, preferably 15- to 25-mer and 55 to 60°C, respectively. Primers of the primer pair may not be equal in length. However, it is preferred that the Tm values of the primers are substantially equal (the difference is usually within 2°C). The Tm values are values calculated by the nearest neighbor method. Examples of the primer pair include a primer pair comprising primers having the nucleotide sequences of SEQ ID NOS: 2 and 3.

PCR is preferably performed in the presence of the probe of the present invention. This enables the Tm analysis without performing any operation of handling the amplification product after completion of the amplification reaction. Tm values of primers and reaction conditions of PCR can be easily adjusted by those skilled in the art depending on the used probe.

A typical example of the composition of the reaction mixture for PCR is as follows.

Table 1

DNA fragments	10^1 to 10^8 molecules/reaction
Primers	200 to 1000 nM
Probe	100 to 1000 nM
Nucleotides	20 to 200 μM each
DNA polymerase	0.01 to 0.03 U/μl
Tris-HCl (pH 8.4 to 9.0)	5 to 20 mM
$MgCl_2$	1.5 to 3 mM
KCl	10 to 100 mM
Glycerol	0 to 20%

(Final fluid volume: 10 to 100 μl)

Further, a typical example of the temperature cycle is as follows, and this temperature cycle is usually repeated 25 to 40 times.

(1) Denaturation at 90 to 98°C for 1 to 60 seconds

(2) Annealing at 60 to 70°C for 10 to 60 seconds

(3) Extension at 60 to 75°C for 10 to 180 seconds

When annealing and extension are performed in one step, conditions of 60 to 70°C for 10 to 180 seconds can be mentioned, for example.

The Tm analysis can be performed in a conventional manner except that fluorescence of the fluorescent dye binding to the probe of the present invention is measured. Fluorescence can be measured by using excitation light having a wavelength suitable for the fluorescent dye and measuring intensity of light of the emission wavelength. The temperature increasing rate in the Tm analysis is usually 0.1 to 1°C per second. Composition of the reaction mixture for Tm analysis is not particularly limited so long as a probe and a nucleic acid having a sequence complementary to the nucleotide sequence of the prove can hybridize to each other. However, the monovalent cation concentration is usually 1.5 to 5 mM, and pH is usually 7 to 9. Because a reaction mixture for an amplification method using a DNA polymerase such as PCR

usually satisfies these conditions, the reaction mixture after the amplification can be used as it is for the Tm analysis.

The B3AR Trp64Arg mutation can be detected on the basis of the results of the Tm analysis in an ordinary manner. The detection in the detection method of the present invention include not only detection of the presence or absence of a mutation, but also quantification of mutant type DNA and determination of the ratio of wild type DNA and mutant type DNA.

<2> Kit of the present invention

The kit of the present invention is a kit used for the second detection method of the present invention. This kit is characterized by including a nucleic acid probe of which end is labeled with a fluorescent dye and in which fluorescence of the fluorescent dye decreases upon hybridization (quenching probe), wherein the nucleic acid probe has a nucleotide sequence starting from the nucleotide number 183 in the nucleotide sequence of SEQ ID NO: 1 and having a length of 8 to 30 nucleotides, and the 5′ end of the probe is labeled with the fluorescent dye, or the nucleic acid probe has a nucleotide sequence ending at the nucleotide number 196 in the nucleotide sequence of SEQ ID NO: 2 and having a length of 7 to 30 nucleotides, and the 3′ end of the probe is labeled with the fluorescent dye.

The quenching probe is as explained above with regard to the probe of the present invention.

The kit of the present invention may include reagents required for amplification of a nucleic acid in the detection method of the present invention, in particular, primers for amplification using a DNA polymerase, in addition to the quenching probe.

In the kit of the present invention, the quenching

probe, primers and other reagents may be separately included, or a part thereof may be provided as a mixture.

Examples

The present invention will be explained more specifically with reference to the following examples.

Example 1

The primers shown in Table 2 were designed on the basis of the nucleotide sequence containing the site of the Trp64Arg mutation of human B3AR gene (SEQ ID NO: 1) so that a region containing the Trp64Arg mutation could be amplified. In Table 2, the positions are indicated with the nucleotide numbers in the nucleotide sequence of SEQ ID NO: 1.

Table 2
Primers

Name	Sequence (5′ → 3′)	mer	Position	SEQ ID NO:
R	gccagcgaagtcacgaacac	20	239-220	3
F	Ggcgctggcggtgc	14	132-145	4

Then, the probes having C at the ends shown in Table 3 were designed. In Table 3, the positions are indicated with the nucleotide numbers in the nucleotide sequence of SEQ ID NO: 1. Further, the capital letters in the nucleotide sequences represent sites of the B3AR Trp64Arg mutation, and (P) at the 3′ ends means being phosphorylated. The probes were labeled with BODIPY (trademark) FL or TAMRA (trademark) in a conventional manner.

Table 3
Probes

Name	Sequence (5′ → 3′)	Mer	Position	SEQ ID NO:
5FL-mt-4-16	(BODIPY FL)-ccatcgccCggactcc-(P)	16	182-197	5
3T-mt-4-16	ccatcgccCggactcc-(TAMRA)	16	182-197	5
5FL-mt-4-19	(BODIPY FL)-ccatcgccCggactccgag-(P)	19	182-200	6
3T-mt-3-19	gtcatcgtggccatcgccC-(TAMRA)	19	172-190	7
3T-mt-2-20	cgtggccatcgccCggactc-(TAMRA)	20	177-196	8
5FL-wt-1-20	(BODIPY FL)-catcgccTggactccgagac-(P)	20	183-202	9
5FL-wt-1-18	(BODIPY FL)-catcgccTggactccgag-(P)	18	183-200	10
5FL-wt-1-16	(BODIPY FL)-catcgccTggactccg-(P)	16	183-198	11
5FL-wt-1-15	(BODIPY FL)-catcgccTggactcc-(P)	15	183-197	12

PCR and Tm analysis were performed by using genomic DNA as a sample and Smart Cycler System (Cephied) under the conditions shown below. The excitation wavelength and the detection wavelength in the Tm analysis were 450 to 495 nm and 505 to 537 nm (BODIPY FL) and 527 to 555 nm and 565 to 605 nm (TAMRA), respectively.

Table 4
Composition of reaction mixture

H_2O	13.2 µL
10 x Gene Taq buffer	2.5 µL
80% Glycerol	6.25 µL
10 mM each dATP, dUTP, dGTP, dCTP	0.5 µL
2 U/µL Uracil-N-glycosylase	0.05 µL
5 µM Probe	1 µL
100 µM Primer F	0.125 µL
100 µM Primer R	0.25 µL
5 U/µL Gene Taq FP	0.125 µL
Sample (0 to 2000 copies)	1 µL
Total	25 µL

Table 5
Reaction conditions
50°C, 2 min
↓
95°C, 2 min
↓
95°C, 1 sec
66°C, 18 sec (50 cycles)
↓
Tm analysis (1°C/sec)

PCR and Tm analysis were performed by using each probe. As a result, only when the probes 3T-mt-2-20, 5FL-wt-1-20, 5FL-wt-1-18, 5FL-wt-1-16 and 5FL-wt-1-15 were used, changes in fluorescence intensity that could be analyzed in Tm analysis were observed. The positions of the probes relative to the nucleotide sequence containing the B3AR Trp64Arg mutation are shown in Figs. 1 and 2. The wild type sequence and mutant type sequence shown in the drawings correspond to the nucleotide numbers 171 to 205 in the nucleotide sequences of SEQ ID NOS: 1 and 2, respectively. Further, in the drawings, F denotes a fluorescent dye. On the basis of the positions shown in Fig. 1 and 2, it is considered that whether the probe can be used for Tm analysis depends on the position of C bound with a fluorescent dye, and the length of the probe is not so important so long as the polymorphism site is included.

In the following, by using the probe 5FL-wt-1-16, sensitivity with respect to the absolute amount of genomic DNA, and reproducibility were examined.

The above method was repeated by using samples containing 0, 20, 200 and 2000 copies of genomic DNA (wild type). The results are shown in Fig. 3. As seen from Fig. 3, it is shown that the genomic DNA can be detected even if 20 copies.

Then, plasmid having the wild type sequence (same as

the above plasmid except that nucleotide number 285 is A in the nucleotide sequence of SEQ ID NO: 1) was prepared. Ten samples (wt/mt) were prepared by mixing the wild type plasmid and the mutant type plasmid. With respect to each of these samples as well as a sample (wt/wt) of only the wild type plasmid and a sample (mt/mt) of only the mutant type plasmid, the above method was repeated. The results are shown in Fig. 4. As seen from Fig. 4, it is shown that the method is excellent in the reproducibility.

Furthermore, by using the probe 3T-mt-2-20 instead of the probe 5FL-wt-1-16, sensitivity with respect to the absolute amount of genomic DNA, and reproducibility were examined in the similar manner. The results are shown in Figs. 5 and 6. As seen from Figs. 5 and 6, it is shown that the sensitivity is high and the reproducibility is excellent.

In Figs. 3 to 6, the vertical axis represents a primary derivative value of fluorescence intensity with an inverted sign (-dF/dt), and the horizontal axis represents temperature (°C).

Industrial Applicability

According to the present invention, a quenching probe effective for detecting the B3AR Trp64Arg mutation is provided, and a method for detecting the B3AR Trp64Arg mutation by using it and a kit therefor are further provided. Because the Tm analysis is completed within several tens of seconds, time required for the detection can be markedly reduced. According to a preferred embodiment of the present invention, wherein amplification of nucleic acid in the presence of the probe and Tm analysis are combined, only the Tm of the probe is analyzed after the amplification of nucleic acid, and therefore it is not necessary to handle the amplification product after completion of the reaction. Accordingly,

there is no risk of contamination with the amplification product. Further, because the detection can be performed with the same equipment as required for the amplification, it is even unnecessary to move a vessel. Therefore, automatization of the method is also easy.